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(54) Title: POLYNUCLEOTIDES AND THEIR USE FOR DETECTING RESISTANCE TO STREPTOGRAMIN A OR TO STREPTOGRAMIN B AND RELATED COMPOUNDS			
(57) Abstract			
<p>The present invention pertains to polynucleotides derived from <i>staphylococcal</i> genes encoding resistance to streptogramin A or to streptogramin B and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting <i>Staphylococcal</i> strains that are resistant to streptogramin A or to streptogramin B and related compounds in a biological sample. In another embodiment, the present invention is directed to the full length coding sequences of the <i>staphylococcal</i> genes encoding for resistance to streptogramin A or to streptogramin B from <i>Staphylococcus</i> and to the polypeptides expressed by these full length coding sequences. Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to characterize any <i>staphylococcal</i> strain carrying genes encoding resistance to streptogramin A or to streptogramin B.</p>			

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POLYNUCLEOTIDES AND THEIR USE FOR DETECTING RESISTANCE TO
STREPTOGRAMIN A OR TO STREPTOGRAMIN B AND RELATED
COMPOUNDS

The present invention pertains to polynucleotides derived from *staphylococcal* genes encoding resistance to streptogramin A or to streptogramin B and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting *Staphylococcal* strains that are resistant to streptogramin A or to streptogramin B and related compounds in a biological sample.

In another embodiment, the present invention is directed to the full length coding sequences of the *staphylococcal* genes encoding for resistance to streptogramin A or to streptogramin B from *Staphylococcus* and to the polypeptides expressed by these full length coding sequences.

Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to characterize any *staphylococcal* strain carrying genes encoding resistance to streptogramin A or to streptogramin B.

The present invention is also directed to diagnostic methods for detecting specific strains of *Staphylococcus* expected to be contained in a biological sample. The diagnostic methods use the oligonucleotide probes and primers as well as the antibodies of the invention.

Streptogramins and related compounds (antibiotics) produced by streptomycetes can be classified as A and B compounds according to their basic primary structures (Cocito, 1979). Compounds of the A group, including streptogramin A (SgA), pristinamycin IIA (PIIA), virginiamycin M, mikamycin A, or synergistin A, are 25 polyunsaturated cyclic macrolactones. Compounds of the B group, including streptogramin B (SgB), pristinamycin B (PIB), virginiamycin S, mikamycin B, and synergistin B, are cyclic peptidic macrolactones (Cocito, 1979). Compounds of both groups, A and B, bind different targets in the peptidyltransferase domain of the 50S

ribosomal subunit and inhibit protein elongation at different steps (Aumercier et al., 1992; Di Giambattista et al., 1989).

A decrease in the dissociation constant of PIB is observed in the presence of PIIA because this latter antibiotic provokes a conformational modification of the bacterial 5 ribosome at the binding sites of these molecules. Thus, A and B compounds, which are bacteriostatic when used separately, act synergistically when combined and become bactericidal, mainly against Gram-positive bacteria.

Natural mixtures such as pristinamycin (Pt), synergistin, virginiamycin and mikamycin, are used orally and topically. A semi-synthetic injectable streptogramin, 10 RP59500, consisting of a mixture of derivatives of A and B compounds (Dalfopristin and Quinupristin, respectively) is currently undergoing *in vivo* experimental and clinical trials (J. Antimicrob. Agents Chemother. 30 (Suppl. A), entire volume, 1992; Entenza et al., 1995; Fantin et al., 1995; Griswold et al., 1996; Torralba et al., 1995). Staphylococcal 15 resistance to synergistic mixtures of A and B compounds (Pt MIC \geq 2 μ g/ml) is always associated with resistance to A compounds (PIIA MIC \geq 8 μ g/ml), but not necessarily with resistance to B compounds (Allignet et al., 1996).

To date, four genes encoding resistance to A compounds have been isolated from staphylococcal and enterococcal plasmids. The genes *vat* (Allignet et al., 1993), *vatB* (Allignet and El Solh, 1995), and *sarA* (Rende-Fournier et al., 1993) encode related 20 acetyltransferases (50.4-58.3 % amino acids), which inactivate streptogramin A and similar compounds. The staphylococcal gene *vga* (Allignet et al., 1992) encodes an ATP-binding protein probably involved in the active efflux of A compounds. Nevertheless, there continues to exist a need in the art for polynucleotides specific for *Staphylococcus* resistant to streptogramin A and/or B and related compounds.

25

SUMMARY OF THE INVENTION

Accordingly, this invention aids in fulfilling this need in the art. In particular, this invention provides a purified peptide comprising an amino acid sequence selected from the group consisting of:

- 5 a) SEQ ID NO: 4 which corresponds to the complete amino acid sequence of Vga B or fragments derived from SEQ ID NO: 4 containing at least 10 amino acids;
- b) SEQ ID NO: 5 which corresponds to the complete amino acid sequence of Vat C or fragments derived from SEQ ID NO: 5 containing at least 10 amino acids;
- 10 c) SEQ ID NO: 6 which corresponds to the complete amino acid sequence of Vgb B or fragments derived from SEQ ID NO: 6 containing at least 10 amino acids;
- d) SEQ ID NO: 7 which corresponds to the complete amino acid sequence of Vgb B;
- 15 e) SEQ ID NO: 8 which corresponds to a fragment of the amino acid sequence of Vga B;
- f) SEQ ID NO: 9 which corresponds to a fragment of the amino acid sequence of Vat C, and
- 20 g) SEQ ID NO: 10 which corresponds to a fragment of the amino acid sequence of Vat C.

This invention additionally provides a purified polynucleotide comprising the nucleotide sequence selected from the group consisting of:

- 25 a) SEQ ID NO: 1 which corresponds to the complete nucleic acid sequence of vga B or fragments derived from SEQ ID NO: 1 containing 15 to 40 nucleotides;
- b) SEQ ID NO: 2 which corresponds to the complete nucleic acid sequence of vat C or fragments derived from SEQ ID NO: 2 containing 15 to 40 nucleotides;
- c) SEQ ID NO: 3 which corresponds to the complete nucleic acid sequence of vgb B or fragments derived from SEQ ID NO: 3 containing 15 to 40 nucleotides;

- d) SEQ ID NO: 11 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 7;
- e) SEQ ID NO: 12 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 8 ;
- 5 f) SEQ ID NO: 13 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 9; and
- g) SEQ ID NO: 14 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 10.

Furthermore, this invention includes a purified peptide comprising the amino acid sequence encoded by the nucleotide sequence selected from the group consisting of :

- 10 a) SEQ ID NO: 1,
- b) SEQ ID NO: 2,
- c) SEQ ID NO: 3,
- d) SEQ ID NO: 11,
- 15 e) SEQ ID NO: 12,
- f) SEQ ID NO: 13, and
- g) SEQ ID NO: 14.

This invention also provides a composition comprising purified polynucleotide sequences including at least one nucleotide sequence selected from the group consisting of polynucleotides, genes or cDNA of *vgaB*, *vaiC*, and *vgbB*, which are useful for the detection of resistance to streptogramin A and/or to streptogramin B and related compounds. This invention further provides a composition comprising purified amino acid sequences including at least an amino acid sequence from a polypeptide encoded by a polynucleotide selected from the group consisting of polynucleotides, genes or cDNA of *vgaB*, *vaiC*, and *vgbB*, which are useful for the detection of resistance to streptogramin A and/or to streptogramin B and related compounds.

In another embodiment, this invention provides a composition of polynucleotide sequences encoding resistance to streptogramins and related compounds, or inducing this resistance in Gram-positive bacteria, wherein the composition comprises a combination of at least two of the following nucleotide sequences: a) a nucleotide sequence encoding

5 an acetyltransferase conferring resistance to streptogramin A and related compounds, b) a nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A and related compounds; and c) a nucleotide sequence encoding a lactonase conferring resistance to streptogramin B and related compounds.

Furthermore, this invention provides a composition of polynucleotide sequences,

10 wherein the sequence encoding a molecule containing ATP binding motifs confers resistance to *Staphylococci* and particularly to *S. aureus*, and wherein the polynucleotide sequence corresponds to a *vgaB* nucleotide sequence represented by SEQ ID NO: 1 or a sequence having at least 70% homology with *vgaB* complete nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 1 under stringent conditions, or to a

15 fragment containing between 20 and 30 nucleotides of SEQ ID NO: 11 or SEQ ID NO: 12, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 4 or with SEQ ID NO: 7 or SEQ ID NO: 8.

Furthermore this invention relates to a composition of polynucleotide sequences, wherein the sequence encoding an acetyltransferase confers resistance to streptogramin

20 A and related compounds in *Staphylococci*, and particularly in *S. cohnii*, and wherein the polynucleotide sequence corresponds to a *vatC* nucleotide sequence represented by SEQ ID NO: 2 or a sequence having at least 70% homology with *vatC* complete nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 2 under stringent conditions, or to a fragment containing between 20 and 30 nucleotides of SEQ ID NO:

25 13 or SEQ ID NO: 14, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 5 or with SEQ ID NO: 9 or SEQ ID NO: 10.

This invention also provides a composition of polynucleotide sequences, wherein the sequence encoding a lactonase confers resistance to streptogramin B and related compounds in *Staphylococci* and particularly in *S. cohnii*, and wherein the polynucleotide sequence corresponds to a *vgbB* nucleotide sequence represented in SEQ ID NO: 3 or

5 a sequence having at least 70% homology with *vgbB* complete nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 3 under stringent conditions, or to a fragment containing between 20 and 40 nucleotides of SEQ ID NO: 3, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 6.

10 The invention also contemplates a composition of polynucleotide sequences, wherein at least a *vaiB* nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds is included in addition to a *vgaB* nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A.

15 Additionally, the invention includes a purified polynucleotide that hybridizes specifically under stringent conditions with a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14.

The invention further includes polynucleotide fragments comprising at least 10

20 nucleotides capable of hybridization under stringent conditions with any one of the nucleotide sequences enumerated above.

In another embodiment of the invention, a recombinant DNA sequence comprising at least one nucleotide sequence enumerated above and under the control of regulatory elements that regulate the expression of resistance to antibiotics of the

25 streptogramin family in a defined host is provided.

Furthermore, the invention includes a recombinant vector comprising the recombinant DNA sequence noted above, wherein the vector comprises the plasmid pIP1633 or plasmid pIP1714.

The invention also includes a recombinant cell host comprising a polynucleotide sequence enumerated above or the recombinant vector defined above.

In still a further embodiment of the invention, a method of detecting bacterial strains that contain the polynucleotide sequences set forth above is provided.

5 Additionally, the invention includes kits for the detection of the presence of bacterial strains that contain the polynucleotide sequences set forth above.

The invention also contemplates antibodies recognizing peptide fragments or polypeptides encoded by the polynucleotide sequences enumerated above.

Still further, the invention provides for a screening method for active antibiotics
10 and/or molecules for the treatment of infections due to Gram-positive bacteria,
particularly staphylococci, based on the detection of activity of these antibiotics and/or
molecules on bacteria having the resistance phenotype to streptogramins.

It is to be understood that both the foregoing general description and the
following detailed description are exemplary and explanatory only and are not restrictive
15 of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be more fully described with reference to the drawings in
which:

20 FIG. 1A and 1B are the restriction maps of the 5.5 kb *Bgl*II fragment and of the
2.4 kb *Hind*III-*Hae*III fragment of pIP1633, respectively. Both fragments confer
resistance to streptogramin A and related compounds. The strategy for sequencing the
2.4 kb *Hind*III-*Hae*III fragment is given in Fig. 1B. Restriction enzyme abbreviations:
Ba, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hind*III; *X*, *Xba*I.

25 FIG. 2 is the nucleotide sequence and deduced amino acid sequence of 2411
nucleotides from pIP1633, which contains the gene *vgaB* of *S. aureus* conferring
resistance to streptogramin A and related compounds. The putative ribosome binding site
(RBS) is underlined. The amino acids are aligned with the second nucleotide of each

codon. Asterisks indicate the in-frame stop codons. The A and B ATP-binding motifs described by Walker et al. (1982) and detected within each of the two ATP-domains are boxed. The conserved motif SGG of the two copies of loop 3 described by Hyde et al. (1990) is underlined. Relevant restriction sites are shown.

5 FIG. 3 is the amino acid sequence alignment of the predicted 60 and 61 kDa proteins encoded by Vga (Allignet et al., 1992, accession No: m90056) and VgaB (FIG.2), respectively. Identical residues are indicated by asterisks and conservative changes are shown by single dots. The A and B motifs of Walker et al. (1982) are in bold type (WA, WB). The conserved motif SGG of the two copies of loop 3 described by
10 Hyde et al. (1990) is underlined.

FIG. 4 is a restriction map of the plasmid pIP1714 carrying the genes *varC* and *vgbB* as well as the genes *pre* and *repB* of *S. cohnii* strain BM10711 resistant to the synergistic mixtures of streptogramins A and B.

15 FIG. 5 is the nucleotide sequence and deduced amino acid sequence of 1727 nucleotide from pIP1714, which contains the gene *vgbB* and *varC* of *S. cohnii*. Relevant restriction sites are shown.

FIG. 6 A, 6B, and 6C represent oligonucleotide primers for hybridization under stringent conditions with *varC*, *vgbB*, and *vgaB* respectively.

FIG. 7 represents SEQ ID NOs: 1-14.

20

DETAILED DESCRIPTION OF THE INVENTION

It has now been determined that bacteria from the *Staphylococcus* genus carry a *vgaB* gene, which encodes a putative ATP-binding protein that confers resistance to streptogramin A and structurally similar compounds. It has also now been determined
25 that bacteria from the *Staphylococcus* genus carry a *vgbB* gene, which encodes a lactonase that confers resistance to streptogramin B and structurally similar compounds, and a *varC* gene, which encodes an acetyltransferase that confers resistance to streptogramin A and structurally similar compounds.

Novel polynucleotides corresponding to the *vgaB*, *vgbB*, and *varC* genes from various strains of *Staphylococcus* have been isolated and sequenced, and it has been surprisingly demonstrated that these new polynucleotides make it possible to design oligonucleotide probes or primers. These polynucleotides include the following:

- 5 a) SEQ ID NO: 1,
- b) SEQ ID NO: 2,
- c) SEQ ID NO: 3,
- d) SEQ ID NO: 11,
- e) SEQ ID NO: 12,
- 10 f) SEQ ID NO: 13, and
- g) SEQ ID NO: 14.

This invention provides specific pairs of oligonucleotide primers or probes that hybridize specifically, under stringent hybridization conditions as defined hereinafter, to the nucleic acid (RNA or DNA) from a particular strain of the *Staphylococcus* genus.

15 These oligonucleotide primers include the following:

- a) Oligo I 5'-AAGTCGACTGACAATATGAGTGGTGG-3'
 Oligo II 5'-CTGCAGATGCCTAACAGCATCGATATCC-3'
- b) Oligo III 5'- ATGAATT CGCAAAT CAGCAAGG -3'
 Oligo IV 5'- TCGTCTCGAGCTCTAGGTCC -3'
- 20 c) Oligo V 5'- CAGCAGTCTAGATCAGAGTGG -3'
 Oligo VI 5'- CATA CGGATCCACCTTTCC -3'.

In a specific embodiment of the present invention, the purified polynucleotides useful for detecting *Staphylococcal* strains can be used in combination in order to detect bacteria belonging to *Staphylococci* in a biological sample. Thus, the present invention 25 also provides detection methods and kits comprising combinations of the purified polynucleotides according to the invention. The purified oligonucleotides of the invention are also useful as primers for use in amplification reactions or as nucleic acid probes.

By "polynucleotides" according to the invention is meant the sequences referred to as SEQ ID NOs: 1, 2, 3, OR 11, 12, 13, 14 and the complementary sequences and/or the sequences of polynucleotides which hybridize to the referred sequences in high stringent conditions and which are used for detecting *staphylococcal* strains carrying a 5 gene encoding resistance to streptogramin A or to streptogramin B.

By "active molecule" according to the invention is meant a molecule capable of inhibiting the activity of the purified polypeptide as defined in the present invention or capable of inhibiting the bacterial culture of *staphylococcal* strains.

Thus, the polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments can 10 be used to select nucleotide primers notably for an amplification reaction, such as the amplification reactions further described.

PCR is described in the U.S. Patent No. 4,683,202 granted to Cetus Corp. The amplified fragments may be identified by agarose or polyacrylamide gel electrophoresis, or by a capillary electrophoresis, or alternatively by a chromatography technique (gel 15 filtration, hydrophobic chromatography, or ion exchange chromatography). The specificity of the amplification can be ensured by a molecular hybridization using as nucleic probes the polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments, oligonucleotides that are complementary to these polynucleotides or fragments thereof, or their amplification products themselves.

20 Amplified nucleotide fragments are useful as probes in hybridization reactions in order to detect the presence of one polynucleotide according to the present invention or in order to detect the presence of a bacteria of *Staphylococcal* strain carrying genes encoding resistance to streptogramin A or streptogramin B, in a biological sample. This invention also provides the amplified nucleic acid fragments ("amplicons") defined herein 25 above. These probes and amplicons can be radioactively or non-radioactively labeled, using for example enzymes or fluorescent compounds.

Preferred nucleic acid fragments that can serve as primers according to the present invention are the following:

polynucleotides of sequence SEQ ID NOs: 1-3 and 11-14; and
polynucleotides having a length from 20 to 30 consecutive
nucleotides from a polynucleotide selected from the group consisting of
polynucleotides of sequences SEQ ID NO: 11 to SEQ ID NO: 14 or from
5 20 to 40 consecutive nucleotides from a polynucleotide of SEQ ID NO:
3

The primers can also be used as oligonucleotide probes to specifically detect a polynucleotide according to the invention.

Other techniques related to nucleic acid amplification can also be used and are
10 generally preferred to the PCR technique. The Strand Displacement Amplification (SDA)
technique (Walker et al., 1992) is an isothermal amplification technique based on the
ability of a restriction enzyme to cleave one of the strands at a recognition site (which is
under a hemiphosphorothioate form), and on the property of a DNA polymerase to
initiate the synthesis of a new strand from the 3' OH end generated by the restriction
15 enzyme and on the property of this DNA polymerase to displace the previously
synthesized strand being localized downstream.

The SDA amplification technique is more easily performed than PCR (a single
thermostated water bath device is necessary), and is faster than the other amplification
methods. Thus, the present invention also comprises using the nucleic acid fragments
20 according to the invention (primers) in a method of DNA or RNA amplification according
to the SDA technique. The polynucleotides of SEQ ID NOs: 1-3 and 11-14 and
their fragments, especially the primers according to the invention, are useful as technical
means for performing different target nucleic acid amplification methods such as:

- TAS (Transcription-based Amplification System), described by Kwok et al. in
25 1989;
- SR (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;
- NASBA (Nucleic acid Sequence Based Amplification), described by Kievitis et
al. in 1991; and

- TMA (Transcription Mediated Amplification).

The polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments, especially the primers according to the invention, are also useful as technical means for performing methods for amplification or modification of a nucleic acid used as a probe, 5 such as:

- LCR (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, who employ a thermostable ligase;
- RCR (Repair Chain Reaction), described by Segev et al. in 1992;
- CPR (Cycling Probe Reaction), described by Duck et al. in 1990; and
- 10 - Q-beta replicase reaction, described by Miele et al. in 1983 and improved by Chu et al. in 1986, Lizardi et al. in 1988, and by Burg et al. and Stone et al. in 1996.

When the target polynucleotide to be detected is RNA, for example mRNA, a reverse transcriptase enzyme can be used before the amplification reaction in order to obtain a cDNA from the RNA contained in the biological sample. The generated cDNA 15 can be subsequently used as the nucleic acid target for the primers or the probes used in an amplification process or a detection process according to the present invention.

Nucleic probes according to the present invention are specific to detect a polynucleotide of the invention. By "specific probes" according to the invention is meant any oligonucleotide that hybridizes with one polynucleotide of SEQ ID NOs: 1-3 and 11-20 14 and which does not hybridize with unrelated sequences. Preferred oligonucleotide probes according to the invention are oligonucleotides I-VI.

In a specific embodiment, the purified polynucleotides according to the present invention encompass polynucleotides having at least 80% homology in their nucleic acid sequences with polynucleotides of SEQ ID NO: 11 to SEQ ID NO: 14, at least 70% 25 identity with SEQ ID NO: 1 to 3. By percentage of nucleotide homology according to the present invention is intended a percentage of identity between the corresponding bases of two homologous polynucleotides, this percentage of identity being purely statistical

and the differences between two homologous polynucleotides being located at random and on the whole length of said polynucleotides.

The oligonucleotide probes according to the present invention hybridize specifically with a DNA or RNA molecule comprising all or part of one polynucleotide

5 among SEQ ID NOs: 1-3 and 11-14 under stringent conditions. As an illustrative embodiment, the stringent hybridization conditions used in order to specifically detect a polynucleotide according to the present invention are advantageously the following:

Prehybridization and hybridization are performed at 68°C in a mixture containing:

- 5X SSPE (1X SSPE is .3 M NaCl, 30 mM tri-sodium citrate
- 5X Denhardt's solution
- 0.5% (w/v) sodium dodecyl sulfate (SDS); and
- 100 µg ml⁻¹ salmon sperm DNA

The washings are performed as follows:

- Two washings at laboratory temperature for 10 min. in the presence of 2 x SSPE and 0.1 % SDS;
- One washing at 68°C for 15 min. in the presence of 1 x SSPE, 1% SDS; and
- One washing at 68°C for 15 min. in the presence of 0.1 x SSPE and 0.1 % SDS.

20 The non-labeled polynucleotides or oligonucleotides of the invention can be directly used as probes. Nevertheless, the polynucleotides or oligonucleotides are generally labeled with a radioactive element (³²P, ³⁵S, ³H, ¹²⁵I) or by a non-isotopic molecule (for example, biotin, acetylaminofluorene, digoxigenin, 5-bromodesoxyuridin, fluorescein) in order to generate probes that are useful for numerous applications.

25 Examples of non-radioactive labeling of nucleic acid fragments are described in the French Patent No. FR 78 10975 or by Urdea et al. or Sanchez-Pescador et al. 1988.

Other labeling techniques can also be used, such as those described in the French patents 2 422 956 and 2 518 755. The hybridization step may be performed in different ways (Matthews et al. 1988). A general method comprises immobilizing the nucleic acid that has been extracted from the biological sample on a substrate (nitrocellulose, nylon, 5 polystyrene) and then incubating, in defined conditions, the target nucleic acid with the probe. Subsequent to the hybridization step, the excess amount of the specific probe is discarded, and the hybrid molecules formed are detected by an appropriate method (radioactivity, fluorescence, or enzyme activity measurement).

Advantageously, the probes according to the present invention can have structural 10 characteristics such that they allow signal amplification, such structural characteristics being, for example, branched DNA probes as those described by Urdea et al. in 1991 or in the European Patent No. 0 225 807 (Chiron).

In another advantageous embodiment of the present invention, the probes described herein can be used as "capture probes", and are for this purpose immobilized 15 on a substrate in order to capture the target nucleic acid contained in a biological sample. The captured target nucleic acid is subsequently detected with a second probe, which recognizes a sequence of the target nucleic acid that is different from the sequence recognized by the capture probe.

The oligonucleotide fragments useful as probes or primers according to the 20 present invention can be prepared by cleavage of the polynucleotides of SEQ ID NOS: 1-3 and 11-14 by restriction enzymes, as described in Sambrook et al. in 1989. Another appropriate preparation process of the nucleic acids of the invention containing at most 200 nucleotides (or 200 bp if these molecules are double-stranded) comprises the following steps:

25 - synthesizing DNA using the automated method of beta-cyanethylphosphoramidite described in 1986;
 - cloning the thus obtained nucleic acids in an appropriate vector;
 and

- purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

A chemical method for producing the nucleic acids according to the invention, which have a length of more than 200 nucleotides (or 200 bp if these molecules are 5 double-stranded), comprises the following steps:

- Assembling the chemically synthesized oligonucleotides having different restriction sites at each end;

- cloning the thus obtained nucleic acids in an appropriate vector; and

10 - purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

The oligonucleotide probes according to the present invention can also be used in a detection device comprising a matrix library of probes immobilized on a substrate, the sequence of each probe of a given length being localized in a shift of one or several bases, 15 one from the other, each probe of the matrix library thus being complementary to a distinct sequence of the target nucleic acid. Optionally, the substrate of the matrix can be a material able to act as an electron donor, the detection of the matrix positions in which hybridization has occurred being subsequently determined by an electronic device. Such matrix libraries of probes and methods of specific detection of a target nucleic acid 20 are described in the European patent application No. 0 713 016, or PCT Application No. WO 95 33846, or also PCT Application No. WO 95 11995 (Affymax Technologies), PCT Application No. WO 97 02357 (Affymetrix Inc.), and also in U.S. Patent No. 5,202,231 (Drmanac), said patents and patent applications being herein incorporated by reference.

The present invention also pertains to a family of recombinant plasmids containing 25 at least a nucleic acid according to the invention. According to an advantageous embodiment, a recombinant plasmid comprises a polynucleotide of SEQ ID NOs: 1-3 and 11-14 or one nucleic fragment thereof. More specifically, the following plasmids are part of the invention: pIP1633 and pIP1714.

The present invention is also directed to the full length coding sequences of the *vgaB*, *vgbB*, and *vatC* genes from *Staphylococci* that are available using the purified polynucleotides according to the present invention, as well as to the polypeptide enzymes encoded by these full length coding sequences. In a specific embodiment of the present

5 invention, the full length coding sequences of the *vgaB*, *vgbB*, and *vatC* genes are isolated from a plasmid or cosmid library of the genome of *Staphylococci* that have been screened with the oligonucleotide probes according to the present invention. The selected positive plasmid or cosmid clones hybridizing with the oligonucleotide probes of the invention are then sequenced in order to characterize the corresponding full length coding sequence,

10 and the DNA insert of interest is then cloned in an expression vector in order to produce the corresponding ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds.

15 A suitable vector for the expression in bacteria and in particular in *E. coli*, is the pQE-30 vector (QIAexpress) that allows the production of a recombinant protein containing a 6xHis affinity tag. The 6xHis tag is placed at the C-terminus of the recombinant polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds, which allows a subsequent efficient purification of the recombinant polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds by passage

20 onto a nickel or copper affinity chromatography column. The nickel chromatography column can contain the Ni-NTA resin (Porath et al. 1975).

25 The polypeptides according to the invention can also be prepared by conventional methods of chemical synthesis, either in a homogenous solution or in solid phase. As an

illustrative embodiment of such chemical polypeptide synthesis techniques the homogenous solution technique described by Houbenweyl in 1974 may be cited.

The polypeptides according to the invention can be characterized by binding onto an immunoaffinity chromatography column on which polyclonal or monoclonal antibodies

5 directed to a polypeptide among the ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds of the invention have previously been immobilized.

10 Another object of the present invention comprises a polypeptide produced by the genetic engineering techniques or a polypeptide synthesized chemically as above described.

The polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related

15 compounds, or lactonase conferring resistance to streptogramin B and related compounds according to the present invention are useful for the preparation of polyclonal or monoclonal antibodies that recognize the polypeptides or fragments thereof. The monoclonal antibodies can be prepared from hybridomas according to the technique described by Kohler and Milstein in 1975. The polyclonal antibodies can be prepared by

20 immunization of a mammal, especially a mouse or a rabbit, with a polypeptide according to the invention that is combined with an adjuvant, and then by purifying specific antibodies contained in the serum of the immunized animal on a affinity chromatography column on which has previously been immobilized the polypeptide that has been used as the antigen.

25 Consequently, the invention is also directed to a method for detecting specifically the presence of a polypeptide according to the invention in a biological sample. The method comprises:

- a) bringing into contact the biological sample with an antibody according to the invention; and
- b) detecting antigen-antibody complex formed.

Also part of the invention is a diagnostic kit for *in vitro* detecting the presence of 5 a polypeptide according to the present invention in a biological sample. The kit comprises:

- a polyclonal or monoclonal antibody as described above, optionally labeled; and
- a reagent allowing the detection of the antigen-antibody complexes formed, wherein the reagent carries optionally 10 a label, or being able to be recognized itself by a labeled reagent, more particularly in the case when the above-mentioned monoclonal or polyclonal antibody is not labeled by itself.

15 Indeed, the monoclonal or polyclonal antibodies according to the present invention are useful as detection means in order to identify or characterize a *Staphylococcal* strain carrying genes encoding resistance to streptogramin A or streptogramin B.

The invention also pertains to:

20 A purified polypeptide or a peptide fragment having at least 10 amino acids, which is recognized by antibodies directed against a polynucleotide sequence conferring resistance to streptogramin and related compounds, corresponding to a polynucleotide sequence according to the invention.

A polynucleotide comprising the full length coding sequence of a *Staphylococcus* 25 streptogramin A and/or B resistant gene containing a polynucleotide sequence according to the invention.

A monoclonal or polyclonal antibody directed against a polypeptide or a peptide fragment encoded by the polynucleotide sequences according to the invention.

A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample comprising:

- 5 a) contacting bacterial DNA of the biological sample with a primer or a probe according to the invention, which hybridizes with a nucleotide sequence encoding resistance to streptogramins;
- b) amplifying the nucleotide sequence using said primer or said probe; and
- c) detecting the hybridized complex formed between said primer or probe with the DNA.

10 A kit for detecting the presence of bacterium having resistance to streptogramin A and/or streptogramin B and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a polynucleotide probe according to the invention; and
- b) reagents necessary to perform a nucleic acid hybridization reaction.

15 A kit for detecting the presence of bacterium having resistance to streptogramin A and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a polynucleotide probe according to the invention; and
- 20 b) reagents necessary to perform a nucleic acid hybridization reaction.

A method of screening active antibiotics for the treatment of the infections due to Gram-positive bacteria, comprising the steps of:

- 25 a) bringing into contact a Gram-positive bacteria having a resistance to streptogramin A or streptogramin B and related compounds and containing the polynucleotide sequences according to the invention with the antibiotic; and

b) measuring an activity of the antibiotic on the bacteria having a resistance to streptogramins and related compounds.

A method of screening for active synthetic molecules capable of penetrating into a bacteria of the family of staphylococci, wherein the inhibiting activity of these molecules 5 is tested on at least a polypeptide encoded by the polynucleotide sequences according to the invention comprising the steps of:

- a) contacting a sample of said active molecules with the bacteria;
- b) testing the capacity of the active molecules to penetrate into the 10 bacteria and the capacity of inhibiting a bacterial culture at various concentration of the molecules; and
- c) choosing the active molecule that provides an inhibitory effect of at least 80% on the bacterial culture compared to an untreated culture.

An *in vitro* method of screening for active molecules capable of inhibiting a 15 polypeptide encoded by the polynucleotide sequences according to the invention, wherein the inhibiting activity of these molecules is tested on at least said polypeptide, said method comprising the steps of:

- a) extracting a purified polypeptide according to the invention;
- b) contacting the active molecules with said purified polypeptide;
- c) testing the capacity of the active molecules, at various 20 concentrations, to inhibit the activity of the purified polypeptide; and
- d) choosing the active molecule that provides an inhibitory effect of at least 80 % on the activity of the said purified polypeptide.

A composition of a polynucleotide sequence encoding resistance to streptogramins and related compounds, or inducing resistance in Gram-positive bacteria, wherein said composition comprises a nucleotide sequence corresponding to the resistance phenotype of the plasmid pIP1633 deposited with the C.N.C.M. under the 5 Accession No. I-1768 and of the plasmid pIP1680 deposited with the C.N.C.M. under the Accession No. I-1767 and of the plasmid pIP1714 deposited with the C.N.C.M. under the number I-1877 on June 18, 1997.

A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample, said method comprising the 10 steps of:

- a) contacting said sample with an antibody according to the invention that recognizes a polypeptide encoded by said polynucleotide sequences; and
- b) detecting said complex.

15 A diagnostic kit for *in vitro* detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a predetermined quantity of monoclonal or polyclonal antibodies according to the invention;
- b) reagents necessary to perform an immunological reaction between the antibodies and a polypeptide encoded by said polynucleotide sequences; and
- c) reagents necessary for detecting said complex between the antibodies and the polypeptide encoded by said polynucleotide sequences.

25 The inhibiting activity of the molecules can be readily evaluated by one skilled in the art. For example, the inhibiting activity of Vga B can be tested by detecting its ATP hydrolysis as described in J.I. Ross et al. (1990), Mol. Microbiol. 4(7):1207-1214

regarding the rate evaluation of the active efflux of antibiotics from a cell. Ross et al. use a different gene, but their gene product functions as a drug efflux pump in the same way as Vga B does.

The inhibiting activity of Vat C can be tested by visualizing the acetylation reaction as described in Allignet et al. (1993) regarding the mechanism of inactivation of A-type compounds conferred by plasmids pIP680 and pIP1156 by thick layer chromatography and NMR.

The inhibiting activity of Vgb B can be tested by detecting the degradation of streptogramin B or a related compound by a microbiological test as described in Allignet et al. (1988).

Plasmids containing the polynucleotides from *Staphylococci*, which confer streptogramin A and/or B resistance, are referred to herein by the following accession numbers:

	<u>Plasmid</u>	<u>Accession No.</u>
15	pIP1714	I- 1877
	pIP1633	I-1768
	pIP680	I-1767

and they have been inserted into vectors which have been deposited at the Collection Nationale de Cultures de Microorganismes ("C.N.C.M.") Institut Pasteur, 28, rue du Docteur Roux, F-75724 Paris Cedex 15, France on June 18, 1997, and August 7, 1996, respectively.

EXAMPLES

Example 1: Cloning of the *vgaB* gene carried by plasmid pIP1633

pIP1633 was isolated from a *S. aureus* transconjugant strain, BM12235, obtained 5 from the donor wild-type *S. aureus* strain, BM3385 (Allignet and EI Solh, 1995). This plasmid carried the *vatB* gene located on a 5.5 *Bg*II fragment, but the other described streptogramin A resistant (SgA') genes were not detected either by hybridization experiments or by PCR (Allignet and EI Solh, 1995). Since the gene *vga* was carried by 10 all the tested staphylococcal plasmids containing the *vat* gene (Allignet et al., 1996), the presence of a *vga*-related gene was suspected in pIP1633. We therefore searched this gene in the recombinant plasmid, pIP1675 (Fig. 1A), containing the *vatB*-5.5 *Bg*II fragment of pIP1633.

First, the 2.4 kb *Hind*III-*Hae*III fragment of pIP1675, which contains only 10 nucleotide from *vatB*, was inserted into plasmid pOX300, and the recombinant plasmid, 15 pIP1717 (Fig. 1B), was introduced by electroporation into the *S. aureus* recipient, RN4220 (Kreiswirth et al., 1983). Plasmid pOX300, also named pOX7, (Dyke and Curnock, 1989), is a hybrid of pUC18 and pEI94ts and replicates in *E. coli* where it confers resistance to ampicillin and to erythromycin, and in *S. aureus* where only 20 resistance to erythromycin is expressed. The *S. aureus* transformants selected on 10 μ g/ml erythromycin were resistant to streptogramin A and related compounds (PIIA MICs = 8-16 μ g/ml). Thus, the 2.4-kb *Hind*III-*Hae*III insert of pIP1717 (Fig. 1B) probably 25 carried a streptogramin A resistance gene and was sequenced. The nucleotide (nucleotide) sequence of this fragment was determined by the dideoxy method (Sanger et al., 1977) with the reagents and the procedure recommended by the suppliers of the T⁷ sequencing kit (Pharmacia International). Arrows indicate the direction and extent of each dideoxy-sequencing reaction. (Fig. 1B).

Example 2: The nucleotide sequence of the *vgaB* gene

The strategy of sequencing on both strands is outlined in Fig. 1 and the sequence of the 2411-bp *Hind*III-*Hae*III insert is given in Fig. 2. An open reading frame (ORF) of 1674 nucleotide extending from nucleotide 682 to 2356 was detected on the same strand as *vaiB* (Fig. 2). The 1674 nucleotide ORF contained an ATG start codon at 5 nucleotide 700 to 702 and was preceded by an 8 nucleotide putative RBS. The ΔG (free energy of association) of interaction of the most stable structure between this putative RBS and the 3'-terminus of the 16S rRNA (MacLaughlin et al., 1981; Moran et al., 1982) calculated according to Tinoco et al. (1973) was -79.4 kJ/mol. The sequence located between the ATG codon and the TAA stop codon at nucleotide 2356 to 2358 may 10 encode a 552 amino acid protein of 61,327 daltons (Da). This putative gene, named *vgaB*, had 58.8 % nucleotide identity with the 1572 bp gene, *vga* (Allignet et al., 1992). The G+C content of *vgaB* (27.2 %) is similar to that of *vga* (29 %), but both values are slightly lower than those of the staphylococcal genome (32 to 36 %) (Kloos and Schleifer, 1986). The nucleotide sequence of *vgaB* has been submitted to the 15 GenBank/EMBL data bank under accession no. u82085.

Example 3: Amino acid sequence analysis of VgaB

The predicted translation product of the *vgaB* gene, VgaB, has a calculated isoelectric point (pI) of 9.60. The hydropathy plot of the VgaB sequence according to 20 the algorithm of Kyte and Doolittle (1982) indicates the protein to be hydrophilic. No similarity to known signal sequences of secreted proteins (von Heijne, 1986; Watson, 1984) was observed.

The amino acid sequence of VgaB was compared with the sequences available in databases (GenBank, release 97.0; EMBL, release 48; SwissProt, release 34). Significant 25 similarity to the ATP-binding domains of numerous ATP-binding Cassette (ABC) proteins was found. The protein giving the best match was Vga (48.3 % identical amino acid, 70.4 % similar amino acid). VgaB and Vga each contain two ATP-binding domains sharing 38.8 % and 39.1 % identical amino acid, respectively. Each of these domains

includes the two ATP-binding motifs described by Walker et al. (1982) (Fig.2). Moreover, the highly conserved SGG sequence of loop 3 found between the two ATP-binding motifs of all investigated ATP-binding proteins (Barrasa et al., 1995; Hyde et al., 1990) was detected in Vga (Allignet et al., 1992) and VgaB (Fig. 2). According to the 5 predicted tertiary structure of ABC model cassette, this loop would be conveniently located to interact with the cell membrane (Hyde et al., 1990). The inter-ATP-binding domain of VgaB is more rich in glutamine (11 Q in 155 amino acid total) than the rest of the sequence of the protein (11 Q/397 amino acid). In contrast, the proportion of glutamine in the inter-ATP-binding domain of Vga is similar to that in the other part of 10 the protein (4 Q/156 amino acid and 14 Q/366 amino acid, respectively). Neither Vga nor VgaB contains hydrophobic transmembrane domains.

The ABC protein MsrA (Ross et al., 1990) is the most similar to Vga and VgaB (35.2 % and 34.4 % identical amino acid, respectively). MsrA confers resistance to erythromycin by increasing the efflux of this antibiotic and to streptogramin B by a 15 mechanism not yet elucidated. MsrA contains two ATP-binding domains with 31.8% amino acid identity and separated by a Q-linker, but no hydrophobic stretches that might be potential membrane spanning domains. The hydrophobic proteins, which are expected to interact with MsrA, are those encoded by similar genes mapping near MsrA in two staphylococcal strains (*smpA*, *smpB*) and also those on the chromosome of the *S. aureus* 20 recipient strain, RN4220 (*smpC*), which does not carry *msrA* (Ross et al., 1995). Ross et al. (1996) have recently reported that SmpC found in the chromosome of RN4220 is not essential for the expression of resistance to erythromycin conferred by MsrA. Thus, further experiments are required to elucidate the mechanisms of resistance conferred by *msrA*, *vga*, or *vgaB* genes.

25 Several ABC transporters, which do not have alternating hydrophobic domains, have been grouped in a subfamily in order to distinguish them from the members of the ABC₂ transporter subfamily, the members of which contain hydrophobic transmembrane domains (Barrasa et al., 1995; Olano et al., 1995; Peschke et al., 1995). Thus, VgaB may

be considered as a new member of the former ABC transporter subfamily. Excluding VgaB, Vga, and MsrA, most of the known ABC transporters that contain two ATP-binding cassettes but no hydrophobic domain(s) were found in lantibiotic or antibiotic producing microorganisms in which they are involved in the active excretion of

5 these molecules. These transporters are encoded by the following genes: *ard1*, an amino-acylnucleoside antibiotic resistance gene from *Streptomyces capreolus* (Barrasa et al., 1995); *carA*, a carbomycin-resistance gene from *Streptomyces thermotolerans* (Schoner et al., 1992); *ImrC*, a lincomycin-resistance gene from *Streptomyces lincolnensis* (Peschke et al., 1995); *oleB*, an oleandomycin-resistance gene from *Streptomyces 10 antibioticus* (Olano et al., 1995); *srmB*, a spiramycin-resistance gene from *Streptomyces ambofaciens* (Geistlich et al., 1992); *tlrC*, a tylosin-resistance gene from *Streptomyces fradiae* (Rosteck et al., 1991); and *petT*, a pepS epidermin-resistance gene from *Staphylococcus epidermidis* (Meyer et al., 1995). The amino acid identity between each of these latter ABC transporters and VgaB is between 23.6 % and 28.7 %.

15 Degenerate primers designed from an analysis of the alignment of the amino acid sequence of Vga and VgaB may be helpful to detect such putative genes by PCR experiments. In the streptogramins producers, the described resistance to these antibiotics consists of streptogramin A inactivation by an as yet unknown mechanism (Fierro et al., 1989), streptogramin B inactivation by a lactonase (Kim et al., 1974) and

20 putative increased export of streptogramin A and streptogramin B by an integral membrane protein, Ptr, exploiting transmembrane proton gradients (Blanc et al., 1995). The NMR spectra of the modified A compounds may be analyzed to verify if their inactivation in the antibiotic producers is similar to that due to the proteins Vat or VatB, which transfer an o-acetyl group to position C14 of PIIA (Allignet et al., 1993).

25 Interestingly, the staphylococcal gene *vgb* (Allignet et al., 1988) found in most plasmids carrying *vga* and *vat* (Allignet et al., 1996), encodes a protein inactivating streptogramin B and related compounds by cleavage of the lactone ring.

Example 4: Distribution and location of the *vgaB* gene in 52 *SgA*^R and independent wild-type staphylococci

A recombinant plasmid containing a fragment of *vgaB*, pIP1705, was constructed

5 to serve as a probe in hybridization experiments under stringent conditions as described previously (Allignet et al., 1996). pIP1705 consists of pUC19 cleaved with *Sa*I and *Pst*I, and an insert of 1051 bp amplified from within *vgaB* by the following primers, which introduce *Pst*I or *Sa*I sites:

Oligo I 5'-AAGTCGACTGACAATATGAGTGGTGG-3'

10 *Sal*

Oligo II 5'-CTGCAGATGCCTAACAGCATCGATATCC-3'

Pstl

The 52 *SgA*⁺ staphylococci investigated (Allignet et al., 1996; El Solh et al., 1980;

15 Loncle et al., 1993) included 10 strains (*S. aureus*, *S. simulans*, *S. haemolyticus*, and *S. cohnii urealyticum*), which harbored 26 to 45 kb plasmids containing *vga*, *vat*, and *vgb*; 21 strains (20 *S. aureus* and one *S. epidermidis*), which harbored 50 to 90 kb plasmids containing *vatB*; 16 strains (12 *S. epidermidis*, three *S. haemolyticus* and one *S. aureus*) with 6 to 15 kb plasmids containing *vga*; one *S. epidermidis* strain which
 20 harbored a plasmid of approximately 20 kb containing *vga-var*; and four *S. aureus* strains, which do not carry nucleotide sequences hybridizing with *vat*, *vatB*, *vga*, or *vgb*. Nucleotide sequences hybridizing with pIP1705 were found only in the 21 large plasmids containing *vatB*. In all these 21 plasmids including pIP1633, the hybridizing nucleotide sequences were detected on a 1.5 kb *Eco*RI fragment, which also hybridized with *vatB*,
 25 suggesting that *vgaB* and *vatB* have conserved relative positions.

Example 5: Results concerning vatC and vgbB genes

The *Staphylococcus cohnii* strain, BM10711, resistant to the synergistic mixtures streptogramin A and streptogramin B and related compounds (pristinamycin, virginiamycin, synergistin, mikamycin, Quinupristin-Dalfopristin) was analyzed. This 5 strain was isolated at Douera hospital (Algeria) where the pristinamycin was frequently used topically. The strain was isolated (Liassine et al., 1997) from a sample provided from a cupboard located in a room occupied by patients suffering from chronic osteomyelitis.

The strain BM10711 harbored several plasmids including pIP1714 (5kb). This 10 plasmid was isolated by electroporation in a *S. aureus* recipient strain, RN4220. The transformant, harboring pIP1714, was selected on BHIA containing 10 µg/ml pristinamycin IIA. Plasmid pIP1714 conferred resistances to streptogramin A and streptogramin B and related compounds.

Plasmid pIP1714 was linearized by cleavage with *Hind*III and cloned in the *Hind*III site of the vector pOX7 also named pOX300 (Dyke et al., 1989, FEMS 15 Microbiol. Lett. 58:209-216). pOX7 results from the cointegration of the *E. coli* vector, pUC18, and *S. aureus* plasmid, pE194. The recombinant plasmid pIP1715 consisting of pOX7 and pIP1714 was used to sequence pIP1714 in its entirety. The gene *vatC* (636 20 nucleotides) encoding an acetyltransferase inactivating streptogramin A and related compounds and the gene *vgbB* (885 nucleotides) encoding a lactonase inactivating streptogramin B and related compounds were found to be carried by this plasmid. The gene *vatC* had 71.7, 62.2 and 64.1 % nucleotides identity with *vat*-related gene, *vatB* and *satA* respectively and the gene *vgbB* presents 69.5 % nucleotides identity with the gene *vgb*.

VatC acetyltransferase exhibits significant similarity with acetyltransferases having 25 the same enzymatic activity and encoded by the genes *vatC*, *vatB*, and *sat* (respectively 69.8, 58.2 and 66.0 % amino acids identity). These proteins belong to a family of xenobiotic acetyltransferases modifying various substrates including streptogramin A and

related antibiotics. VgbB lactonase exhibits as well significant similarity with Vgb inactivating streptogramin B and related (67.0 % amino acids identity).

The two other genes carried by pIP1714 are *pre* and *repB*, encoding proteins involved in mobilization and replication, respectively. These two genes are homologous 5 to those carried by the staphylococcal plasmid, pUB110 (McKenzie et al., 1986, Plasmid 15:93-103). Moreover, as reported in Figure 5, the intergenic sequences of pIP1714 delimited by *valC* and *repB* also exhibited significant similarities with pUB110.

Example 6: Plasmid DNA isolation from PIIA^R staphylococci

10 The staphylococci were grown after overnight incubation at 37°C in 200 ml BHI containing 10 µg/ml of PIIA. After 15 min centrifugation at 8000 rpm, the pellet was resuspended in 25 ml TES (Tris 50 mM, EDTA 1 mM, saccharose 7%). After adding 150 µg of lysostaphin, the mixture was incubated 30 min at 37°C. Then, 2ml of SDS 20% and 6 ml of EDTA 0.25 M were added and the suspension was incubated 15 min at 15 37°C. 8 ml of NaCl 5M were added and the mixture was kept 90 min at +4°C. After 30 min centrifugation at 8000 rpm, the supernatent was incubated 15 min at 37°C with 5 µg of Rnase (Boehringer). 10 µg of Proteinase K were added and the suspension was incubated 15 min at 65°C. DNA was precipitated using isopropanol (0.6 V for 1 V of DNA solution). After 30 min centrifugation at 8000 g, the pellet was washed with 10 ml 20 ethanol 70%. The washed DNA was dried at 56°C, dissolved in 10 ml water and purified by dye-buoyant density centrifugation (ethidium bromide - cesium chloride). The extrachromosomal band was collected. After removing ethidium bromide, the solution of plasmid DNA was dialysed using TE buffer (Tris, 10 mM, EDTA 1 mM, pH 7).

25 **Example 7: Plasmid DNA isolation from E. coli**

Cf. QIAfilter plasmid maxi protocol for large-scale preparations and QIAprep Spin plasmid kit protocol for mini-preparations.

Quiagen GmbH and Quiagen Inc. (Hilden, Germany)

	- Plasmid maxi kit	Ref	:	12262
5	- Miniprep kit	Ref	:	27104

Example 8: Transformation by electroporation of the *S. aureus* recipient strain, RN4220

10 **1 - Preparation of cells**

200ml of BHI was inoculated with 20ml of an overnight culture of RN4220 (Kreiswirth *et al.*, *Nature* 1983, 306:709-712) and incubated at 37°C with shaking. When the OD reached 0.4 at 600 nm, the suspension was kept in ice. The pellet was washed three times with 20 ml of cold Hepes buffer (saccharose 9.31 % - Hepes 0.19 % 15 - pH. 7.4). The pellet was resuspended in 2.5 ml of Hepes buffer containing 10% glycerol. Aliquots of 100 µl cell suspension ($3.10^{10}/\text{ml}$) were stored at -80°C.

2 - **Electroporation**

20 After thawing at room temperature, the 100 µl aliquot of cells was kept in ice. After adding 10 µl of a solution containing 1 µg of plasmid DNA, the mixture was transferred to a cold 0.2 cm electroporation cuvette. The Gene Pulser (BioRad) was set at 25 uF and 2.5 KV and the Pulse Controller to 100Ω. This produced a pulse with a constant time of 2.3 to 2.5 m sec. The cuvette was removed from the chamber and 1 ml of SOC (2% bactotryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM 25 MgSO₄, 20 mM glucose) was added. The cell suspension was transferred in a propylene tube and incubated with shaking at 37°C for 1 hr. The suspension was then plated on selective medium, which consisted of BHIA containing 10 µg/ml erythromycin or 10

μ g/ml of PIIA. The plates were incubated 48 h at 37°C and the transformants isolated on selective medium. The further studies were carried out on a single isolated colony.

Example 9: Polymerase chain reaction

5 DNA was amplified by PCR in a Crocodile II thermal cycler (Appligène) with approximately 10ng of cellular DNA or 1ng of plasmid DNA. The reaction mixture contained 0.6 μ M of each oligonucleotide serving as primer, 200 μ M of each deoxynucleotide triphosphate, 2.5 U of *Taq* DNA Polymerase (Amersham, Int.), and 1 x buffer (Amersham, Int.). The final reaction volume was adjusted to 100 μ l with H₂O
10 and the sample was then covered by 50 μ l of heavy white mineral oil (Sigma Chemical Co, St. Louis, Missouri).

PCR experiments were carried out at high or low stringency, depending on the primers used. At high stringency, the PCR was performed with a precycle of 3 min at 95°C and 2 min at 60°C, 30 cycles of 20 sec at 72°C, 20 sec at 95°C, 20 sec at 60°C
15 followed by a cycle of 1 min at 72°C. At low stringency, the PCR was performed with a precycle of 5 min at 95°C, 35 cycles of 2 min at 40°C, 1 min 30 sec at 72°C, 30 sec at 95°C followed by a cycle of 4 min at 40°C and 12 min at 72°C. The oligonucleotides used at high stringency are indicated in the Table below.

PRIMER	
<i>vgaB</i>	Oligo I 5'- <u>AAGTCGACT</u> GACAATATGAGTGTTGG-3' <i>Sal</i> I Oligo II 5'- <u>CTGCAGATGCCTAACAGCATCGATATCC</u> -3' <i>Pst</i> I
<i>vatC</i>	Oligo III 5'- AT <u>GAATTC</u> CGCAAATCAGCAAGG -3' <i>Eco</i> RI Oligo IV 5'- TCGTCTC <u>CGAGCT</u> CTAGGTCC -3' <i>Sac</i> I
<i>vgbB</i>	Oligo V 5'- CAGCAG <u>TCTAGAT</u> CAGAGTGG -3' <i>Xba</i> I Oligo VI 5'- CATA <u>CGGATCC</u> ACCTTTCC -3' <i>Bam</i> H1

Example 10: Labelling of DNA probes

Plasmid DNA was labelled with [α -³²P]dCTP (110 Tbq mmol⁻¹) by the random printing technique using the Megaprime DNA labelling system (Amersham).

5

Example 11: Blotting and hybridization

Hybond-N+membranes (Amersham) were used for blotting. DNA was transferred from agarose gels to the membranes by the capillary blotting method of Southern Blotting. DNA was denatured and fixed to the membranes according to the protocol 10 described in the handbook user of Hybond-N+ membranes.

Prehybridization and hybridization were done at 68°C in a mixture containing 5X SSPE (1X SSPE is 0.3 M NaCl, 30 mM tri-sodium citrate), 5X Denhardt's solution, 0.5% (w/v) SDS, and 100 µg ml⁻¹ salmon sperm DNA. The membranes containing DNA transferred from agarose gels were treated with 10 ng ml⁻¹ radiolabeled DNA probe.

5 Washing was started with two successive immersions in 2X SSPE, 0.1% SDS, at room temperature for 10 min, followed by one immersion in 1X SSPE, 0.1% SDS, at 68°C for 15 min, and finally by one immersion in 0.1 X SSPE, 0.1% SDS, at 68°C for 15 min. The washed blots treated with the radiolabeled probe were exposed to Fuji RX film at -70°C.

10 Example 12: Nucleotides sequence determination

For *vatC* and *vgbB*, the sequencing reaction was performed by PCR amplification in a final volume of 20 µl using 500 ng of plasmid DNA, 5-10 pmoles of primer and 9.5 µl of DyeTerminators premix according to Applied Biosystems protocol. After heating to 94°C for 2 min, the reaction was cycled as the following: 25 cycles of 30s at 94°C, 30s

15 at 55°C, and 4 min at 60°C (9600 thermal cycler Perkin Elmer). Removal of excess of DyeTerminators were performed using Quick Spin columns (Boehringer Mannheim). The samples were dried in a vacuum centrifuge and dissolved with 4 µl of deionized formamide EDTA pH 8.0 (5/1). The samples were loaded onto an Applied Biosystems 373A sequencer and run for 12 h on a 4.5% denaturing acrylamide gel.

- Primers used for sequencing the following genes:

◦ *vatC*

5'-GAAATGGTGGGAGAAGCATAAC-3'	5'-CAGCAATCGCGCCGTTG-3'
5'-AATCGGCAGAATTACAAACG-3'	5'-CGTTCCCAATTCCGTGTTACC-3'

◦ *vgbB*

5'-GTTTCTATGCTGATCTGAATC-3'	5'-GTCGTTGTAATTCTGCCGATT-3'
5'-GGTCTAAATGGCGATATATGG-3'	5'-TTCGAATTCTTTATCCTACC-3'

5

For *vgaB*. DNA was sequenced according to the instructions provided by the T7SequencingTm kit from Pharmacia Biotech (Uppsala, Sweden), procedures C and D.

- Primers used for sequencing the following genes:

◦ *vgaB*

5'-GCTTGGCAAAAGCAACC-3'	5'-TGAATATAGGATAG-3'
5'-TTGGATCAGGGCC-3'	5'-CAATTAGAAGAACAC-3'
5'-CAATTGTTCAGCTAGG-3'	5'-GAATTCAATTCTATGG-3'
5'-TACACCATTGTTACC-3'	5'-CAAGGAATGATTAAGCC-3'
5'-GATTCAAGATGTTCCC-3'	5'-TCATGGTCGCAATG-3'
5'-GTTGCTTCGTAGAACG-3'	5'-GTTATGTCATCCTC-3'
5'-GGTCATCTACGAGC-3'	5'-GGATATCGATGCTG-3'
5'-GCCAACTCCATT-3'	5'-CCTAGCTGAACAATTG-3'
5'-GAAGGTGCCTGATCC-3'	5'-ATACTAGAAATGC-3'

Example 13: DNA cloning

A standard protocol was followed for cloning into the vector pOX7, also named pOX300, the 2.4 kb *Hind*III-*Hae*III fragment of pIP1633 carrying *vgaB* (Fig. 1) and the plasmid pIP1714 carrying *vatC* and *vgbB* (Fig. 4), linearized by cleavage with *Hind*III. The vector DNA (10-20 µg) and the plasmids used in cloning experiments were cleaved with the appropriate restriction enzymes (30 Units) and purified by GeneClean Kit (Bio 101, La Jolla, Calif.). To avoid religation, the vector cleaved with a single enzyme was 10 dephosphorylated by 30 min incubation at 37°C with 5 Units of alkaline phosphatase. Ligation was carried out in a total reaction volume of 10 µl containing 0.1 µg of the vector, 0.1 µg of the plasmid, 0.5 mM ATP, 1 X T4 DNA ligase buffer and 0.1 Weiss Unit of T4 DNA ligase. After overnight incubation at 16°C, 1 to 2 µl of the ligation mixture are used for transforming competent *E. coli* and the transformants were selected 15 on solid media containing 100 µg/ml of ampicillin.

Example 14: Susceptibility to antimicrobial agents

Susceptibility to antimicrobial agents was determined with a disk diffusion assay and commercially available disks (Diagnostic Pasteur). Additional disks prepared in our laboratory contained streptogramin A (20 µg) or streptogramin B (40 µg).

5 - NCCLS: Performance standards for antimicrobial disk susceptibility test, 1984, Approved standard M2-A3, 4:369-402.

 - ECCLS: Standard for antimicrobial susceptibility testing by diffusion methods, 1985, ECCLS Document, 5:4-14.

Minimal inhibitory concentrations (MICs) of antibiotics were determined by serial 10 twofold dilutions of antibiotics in MHA (Ericson H.M. and S.C. Sherris, *ActaPathol. Microbiol. Scand.*, 1971, Suppl. 217:Section B).

Despite the relatively low frequency of detection of SgA^R staphylococci (1-10%) (Loncle et al., 1993; Allignet et al., 1996), four genes encoding resistance to streptogramin A have been detected and other resistance gene(s) are suspected to be 15 carried by staphylococci. Surprisingly, the present and previous studies (Allignet et al., 1996) indicate that staphylococcal plasmids carrying two genes encoding streptogramin A resistance by two distinct mechanisms (inactivation by acetyltransferases and increased efflux) are widespread among staphylococci (32 of the 48 plasmids investigated).

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The following publications have been cited herein. The entire disclosure of each publication is relied upon and incorporated by reference herein.

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WHAT IS CLAIMED IS:

1. A purified polynucleotide comprising the nucleotide sequence selected from the group consisting of SEQ ID NO: 1 or a fragment derived from SEQ ID NO: 1 containing 15 to 40 nucleotides, SEQ ID NO: 11, and SEQ ID NO: 12.
2. A purified peptide comprising the amino acid sequence encoded by the nucleotide sequence selected from the group consisting of SEQ ID NO: 1 or a fragment derived from SEQ ID NO: 1 containing 15 to 40 nucleotides, SEQ ID NO: 11, and SEQ ID NO: 12.
- 10 3. A purified peptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 4 or a fragment derived from SEQ ID NO: 4 containing at least 10 amino acids, SEQ ID NO: 7, and SEQ ID NO: 8.
4. A purified polynucleotide comprising the nucleotide sequence of SEQ ID NO: 3 or a fragment derived from SEQ ID NO: 3 containing 15 to 40 nucleotides.
- 15 5. A purified peptide comprising the amino acid sequence encoded by the nucleotide sequence selected of SEQ ID NO: 3 or a fragment derived from SEQ ID NO: 3 containing 15 to 40 nucleotides.
6. A purified peptide comprising the amino acid sequence of SEQ ID NO: 6 or a fragment derived from SEQ ID NO: 6 containing at least 10 amino acids.
- 20 7. A purified polynucleotide comprising the nucleotide sequence selected from the group consisting of SEQ ID NO: 2 or a fragment derived from SEQ ID NO: 2 containing 15 to 40 nucleotides, SEQ ID NO: 13, and SEQ ID NO: 14.
8. A purified peptide comprising the amino acid sequence encoded by the nucleotide sequence selected from the group consisting of SEQ ID NO: 2 or a fragment derived from SEQ ID NO: 2 containing 15 to 40 nucleotides, SEQ ID NO: 13, and SEQ ID NO: 14.

9. A purified peptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 5 or a fragment derived from SEQ ID NO: 6 containing at least 10 amino acids, SEQ ID NO: 9, and SEQ ID NO: 10.

10. A composition of polynucleotide sequences useful for the detection of 5 resistance to streptogramins A and/or B and related compounds comprising at least a nucleotide sequence selected from the group consisting of the polynucleotide or gene or cDNA *vrgaB*, *vgbB*, and *varC*.

11. A composition of polynucleotide sequences useful for the detection of 10 resistance to streptogramin A or streptogramin B and related compounds comprising at least an amino acid sequence from a polypeptide encoded by a polynucleotide selected from the group consisting of the polynucleotide, gene, or cDNA *vgaB*, *vgbB*, and *varC*.

12. A composition of polynucleotide sequences encoding resistance to 15 streptogramins and related compounds, or inducing streptogramin resistance in Gram-positive bacteria, wherein said composition comprises at least a nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A and related compounds and at least a nucleotide sequence selected from the following sequences:

20 a) a nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds; and

b) a nucleotide sequence encoding a lactonase conferring resistance to streptogramin B and related compounds.

13. A composition of polynucleotide sequences encoding resistance to 25 streptogramins and related compounds, or inducing streptogramin resistance in Gram-positive bacteria, wherein said composition comprises at least a nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds and at least a nucleotide sequence selected from the following sequences:

- a) a nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A and related compounds; and
- b) a nucleotide sequence encoding a lactonase conferring resistance to streptogramin B and related compounds.

5 14. A composition of polynucleotide sequences encoding resistance to streptogramins and related compounds, or inducing streptogramin resistance in particular in Gram-positive bacteria, wherein said composition comprises at least a nucleotide sequence encoding a lactonase conferring resistance to streptogramin B and related compounds and at least a nucleotide sequence selected from the following sequences:

10 a) a nucleotide sequence encoding a material containing ATP binding motifs conferring resistance to streptogramin A and related compounds; and

b) a nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds.

15 15. A composition of polynucleotide sequences according to any one of the claims 12 to 14, wherein the polynucleotide sequence encoding a molecule containing ATP binding motifs confers resistance to streptogramin A and related compounds in *Staphylococcus*, and wherein the polynucleotide corresponds to a *vgaB* nucleotide sequence represented in the SEQ ID NO: 1 or to a nucleotide sequence having at least

20 70 % of identity with the *vgaB* complete nucleotide sequence, or corresponds to a polynucleotide hybridizing with said SEQ ID NO: 1 under stringent conditions, or to a fragment containing between 20 and 30 nucleotides of SEQ ID NO: 11 or SEQ ID NO: 12 or, wherein the polynucleotide sequence encodes a polypeptide having at least 60 % homology with the complete SEQ ID NO: 4 or 80 % with SEQ ID NO: 11 or SEQ ID NO: 12.

25 16. A composition of claim 15, wherein the polynucleotide sequence encoding a molecule containing ATP binding motifs confers resistance to streptogramin A and related compounds in *Staphylococcus aureus*.

17. A composition of polynucleotide sequences according to any one of the claims 12 to 14, wherein the polynucleotide sequence encoding an acetyltransferase confers resistance to streptogramin A and related compounds in *Staphylococcus*, and wherein the polynucleotide corresponds to a *varC* nucleotide sequence represented in the

5 SEQ ID NO: 2 or to a nucleotide sequence having at least 70 % of identity with the *varC* complete nucleotide sequence, or corresponds to a polynucleotide hybridizing with said SEQ ID NO: 2 under stringent conditions or to a fragment of SEQ ID NO: 2 containing between 20 and 30 nucleotides, or to SEQ ID NO: 13 or SEQ ID NO: 14, or wherein the polynucleotide sequence encodes a polypeptide having at least 60 % homology with the

10 complete SEQ ID NO: 5 or 80 % with SEQ ID NO: 13 or SEQ ID NO: 14.

18. A composition of claim 17, wherein the polynucleotide sequence encoding an acetyltransferase confers resistance to streptogramin A and related compounds in *Staphylococcus cohnii*.

19. A composition of polynucleotide sequences according to any one of the claims 12 to 14, wherein the polynucleotide sequence encoding a lactonase confers resistance to streptogramin B and related compounds in *Staphylococcus*, and wherein the polypeptide corresponds to a *vgbB* nucleotide sequence represented in the SEQ ID NO: 3 or to a nucleotide sequence having at least 70 % of identity with the *vgbB* complete nucleotide sequence, or corresponds to a polynucleotide hybridizing with said SEQ ID NO: 3 under stringent conditions or to a fragment of said SEQ ID NO: 3 containing 20 to 40 nucleotides, or wherein the polynucleotide sequence encodes a polypeptide having at least 60 % homology with the complete SEQ ID NO: 6.

20. A composition of claim 19, wherein the polynucleotide sequence encoding a lactonase confers resistance to streptogramin A and related compounds in

25 *Staphylococcus cohnii*.

21. A composition of polynucleotide sequences according to claim 12 or 13, wherein said composition comprises at least one nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A and related

compounds and at least one nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds.

22. A composition of polynucleotide sequences according to claim 21, wherein said composition comprises at least a *vgaB* nucleotide sequence encoding a molecule 5 containing ATP binding motifs conferring resistance to streptogramin A and related compounds and at least a *varB* nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds.

23. A purified polynucleotide that hybridizes specifically under stringent conditions with a polynucleotide sequence selected from the group consisting of SEQ ID 10 NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14.

24. A polynucleotide fragment comprising at least 10 nucleotides, that hybridizes under stringent conditions with a sequence according to any one of claims 1- 9 or 23 or the polynucleotide complementary fragment thereof.

15 25. A polynucleotide fragment of claim 12, wherein said fragment corresponds to at least one of the following sequences:

- Oligo I 5'-AAGTCGACTGACAATATGAGTGTTGG-3'

- Oligo II 5'-CTGCAGATGCCTAACAGCATCGATATCC-3'.

26. A polynucleotide fragment of claim 12, wherein said fragment corresponds 20 to at least one of the following sequences:

- Oligo III 5'- ATGAATTGCAAATCAGCAAGG -3'

- Oligo IV 5'- TCGTCTCGAGCTCTAGGTCC -3'.

27. A polynucleotide fragment of claim 12, wherein said fragment corresponds to at least one of the following sequences:

25 - Oligo V 5'- CAGCAGTCTAGATCAGAGTGG -3'

- Oligo VI 5'- CATACGGATCCACCTTTCC -3'.

28. A recombinant DNA sequence comprising at least a nucleotide sequence according to any one of claims 1, 4, or 7 under the control of regulatory elements that

regulate the expression of resistance to antibiotics of the streptogramin family in a defined host.

29. A recombinant vector comprising the DNA sequence of claim 31, wherein the vector comprises a plasmid pIP1633 deposited with the C.N.C.M. under the Accession 5 No. I-1768 or a plasmid pIP1714 deposited with the C.N.C.M. under the number I-1877 on June 18, 1997.

30. A recombinant cell host comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO 10 3, SEQ ID NO: 2, SEQ ID NO: 13, and SEQ ID NO: 14, or the recombinant vector of claim 32.

31. A purified polypeptide or a peptide fragment having at least 10 amino acids, which is recognized by antibodies directed against a polynucleotide sequence conferring resistance to streptogramin and related compounds, corresponding to a polynucleotide sequence according to any one of claims 1, 4, or 7.

15 32. A polynucleotide comprising the full length coding sequence of a *Staphylococcus* streptogramin A and/or B resistant gene containing a polynucleotide sequence according to any one of claims 1, 4, or 7

33. A monoclonal or polyclonal antibody directed against a polypeptide or a peptide fragment encoded by the polynucleotide sequences according to any one of claims 20 1, 4, or 7.

34. A method of detecting the presence of bacterium harboring the polynucleotide sequences according to any one of claims 1, 4, or 7 in a biological sample comprising:

25 a) contacting bacterial DNA of the biological sample with a primer or a probe according to any one of claims 24-27, which hybridizes with a nucleotide sequence encoding resistance to streptogramins;

- b) amplifying the nucleotide sequence using said primer or said probe; and
- c) detecting the hybridized complex formed between said primer or probe with the DNA.

5 35. A kit for detecting the presence of bacterium having resistance to streptogramin A and/or streptogramin B and harboring the polynucleotide sequences according to any one of the claims 1, 4, or 7 in a biological sample, said kit comprising:

- a) a polynucleotide probe according to any one of claims 24-27; and
- 10 b) reagents necessary to perform a nucleic acid hybridization reaction.

36 A kit for detecting the presence of bacterium having resistance to streptogramin A and harboring the polynucleotide sequences according to claim 1 or claim 7 in a biological sample, said kit comprising:

- 15 a) a polynucleotide probe according to claim 24-26; and
- b) reagents necessary to perform a nucleic acid hybridization reaction.

37. A method of screening active antibiotics for the treatment of the infections due to Gram-positive bacteria, comprising the steps of:

- 20 a) bringing into contact a Gram-positive bacteria having a resistance to streptogramin A or streptogramin B and related compounds and containing the polynucleotide sequences according to any one of the claims 1, 4, or 7 with the antibiotic; and
- b) measuring an activity of the antibiotic on the bacteria having a resistance to streptogramins and related compounds.

25 38. A method of screening for active synthetic molecules capable of penetrating into a bacteria of the family of staphylococci, wherein the inhibiting activity of these

molecules is tested on at least a polypeptide encoded by the polynucleotide sequences according to any one of claims 1, 4, or 7 comprising the steps of:

- a) contacting a sample of said active molecules with the bacteria;
- 5 b) testing the capacity of the active molecules to penetrate into the bacteria and the capacity of inhibiting a bacterial culture at various concentration of the molecules; and
- c) choosing the active molecule that provides an inhibitory effect of at least 80% on the bacterial culture compared to an untreated
- 10 culture.

39. An *in vitro* method of screening for active molecules capable of inhibiting a polypeptide encoded by the polynucleotide sequences according to any one of claims 1, 4, or 7, wherein the inhibiting activity of these molecules is tested on at least said polypeptide, said method comprising the steps of:

- 15 a) extracting a purified polypeptide according to claim 31;
- b) contacting the active molecules with said purified polypeptide;
- c) testing the capacity of the active molecules, at various concentrations, to inhibit the activity of the purified polypeptide; and
- 20 d) choosing the active molecule that provides an inhibitory effect of at least 80 % on the activity of the said purified polypeptide.

40. A composition of a polynucleotide sequence encoding resistance to streptogramins and related compounds, or inducing resistance in Gram-positive bacteria, wherein said composition comprises a nucleotide sequence corresponding to the resistance phenotype of the plasmid pIP1633 deposited with the C.N.C.M. under the Accession No. I-1768 and of the plasmid pIP1680 deposited with the C.N.C.M. under the Accession No. I-1767 and of the plasmid pIP1714 deposited with the C.N.C.M. under the number I-1877 on June 18, 1997.

41. A method of detecting the presence of bacterium harboring the polynucleotide sequences according to claim 1 or claim 7 in a biological sample, said method comprising the steps of:

- 5 a) contacting said sample with an antibody according to claim 36 that recognizes a polypeptide encoded by said polynucleotide sequences; and
- b) detecting said complex.

42. A diagnostic kit for *in vitro* detecting the presence of bacterium harboring the polynucleotide sequences according to claim 1 or claim 7 in a biological sample, said kit 10 comprising:

- 15 a) a predetermined quantity of monoclonal or polyclonal antibodies according to claim 33;
- b) reagents necessary to perform an immunological reaction between the antibodies and a polypeptide encoded by said polynucleotide sequences; and
- c) reagents necessary for detecting said complex between the antibodies and the polypeptide encoded by said polynucleotide sequences.

Figure 1A

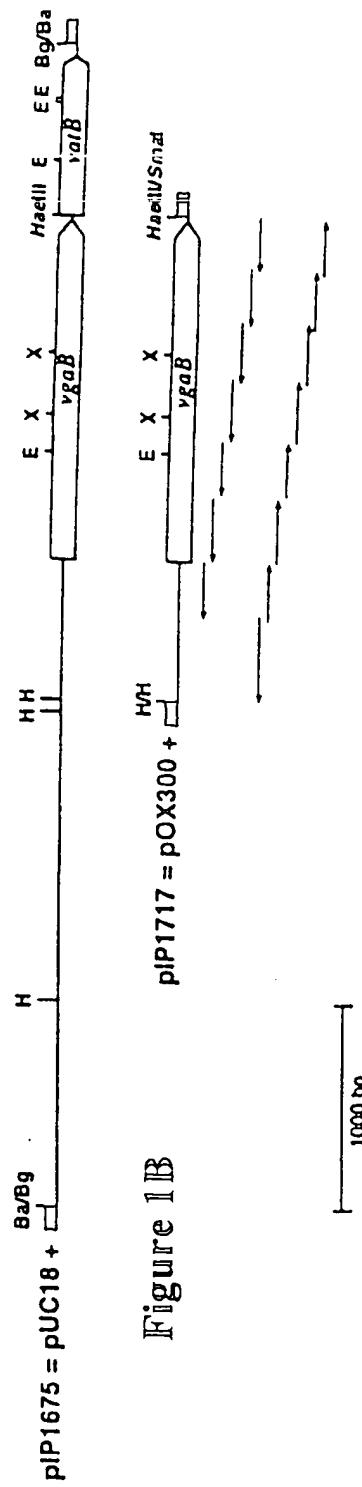
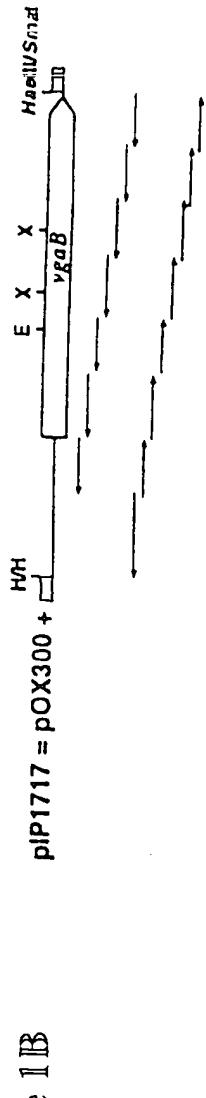


Figure 1B

pIP1717 = pOX300 + H



2/12

Figure 2

24

Figure 2 (cont.)

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WA	-MLKIDMKNVVKYYADKLJLN1KELK1YSGDKIG1IVGRNGVGR1TLLK1IKGLIE1DEGN MK1MLEGLNIKHYVQDRLLN1NRLK1YQND1R1GL1G1NGSGK1TLLH1LYKK1V1P1E1G1- .	59
	loop 3	
V _{ga} B V _{ga}	III1SEKTT1KY1S1Q1L1E1P1H1S1K1I1D1G1K1Y1A1S1F1Q1V1E1N1K1D1N1M1S1G1E1K1T1F1R1K1F1L1A1E1C1G1F1Q1O1C1S1 -IVKQFSH1C1L1P1Q1L1E1S1- .	119 99
WB	LMLVDEPTSNLDIEGIELITNTFKEYRDTFLUVSHDRIFLDQVCTK1F1E1H1E1N1G1Y1I1F1E1R1F1I1G1 LLLADEPPTNLDDNN1IEKLEQD1K1W1G1F1I1V1S1H1D1R1F1D1N1L1C1T1I1E1D1G1R1T1E1Y1K1G .	179 159
V _{ga} B V _{ga}	NY1TN1Y1E1Q1K1E1M1L1R1K1Q1E1Y1K1Y1N1S1K1R1K1Q1L1E1Q1A1K1L1K1E1N1K1A1Q1M1K1P1S1K1T1M1G1T1S1E1S1- R NY1SN1Y1E1Q1K1E1L1R1H1E1L1E1Y1K1Y1E1K1E1K1R1L1E1K1L1E1S1G1K1K .	237 218
V _{ga} B V _{ga}	I1W1K1M1Q1H1A1T1K1Q1K1E1R1N1H1V1E1K1E1L1P1S1K1M1D1P1N1R1Q1F1H1G1R1N1V1S1L1K1V1 G1A1K1P1Y1F1A1G1K1Q1K1L1R1K1T1V1K1S1E1T1R1L1E1K1L1E1S1V1N1L1E1S1V1R1T1I1G1E1D1V .	297 278

Figure 3

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V_{ga}B V_{ga}	SIKFNNQFLWRDASFVIKGGEKVAIIIGNNGVGRKTTLLKLILEKVESTVIISPSVKIGYVSQ SGTIEGRVLWKAKSFSIRGGDKMAIIGSNGTGRKTFICKIVHGNPGISLSPSVKIGYFSQ
loop 3:	
V_{ga}B V_{ga}	NLDVLQSHKSILENTMSIQQETIARIIVLARHFTYRNDVHKEINVLSGGEQIQIVAFAKL KIDTLELDKSILENVQSSSQNETLIRITLARMEFFFRDDVYKPISVLSSGGERVVALTKV
WB	
V_{ga}B V_{ga}	FVSDCNTLILDEPTNLDIDAVEALEELLITYEGVVLFAASHDKKFIQNLAEQLLIIENK FLSEVNNTVLVLDDEPTNLDMEAIEAFESLLKEYNGSIIIFVSHDRKFIEKVATRIMTIDNKE
V_{ga}B V_{ga}	
V_{ga}B V_{ga}	VKKFEGTYIEYLKIKDKPKLNTNEKELKEKAMILEQISSLLSKISMEEENEENKELDEK IKIFDGTY-EQFRQAEKPTRNIKE---DKKLLETKITEVLSRLSIEPSEE---LEQE
V_{ga}B V_{ga}	
V_{ga}B V_{ga}	YKLKLKELKSLNQII FQNLINKERNLDK--

Figure 3 (cont.)

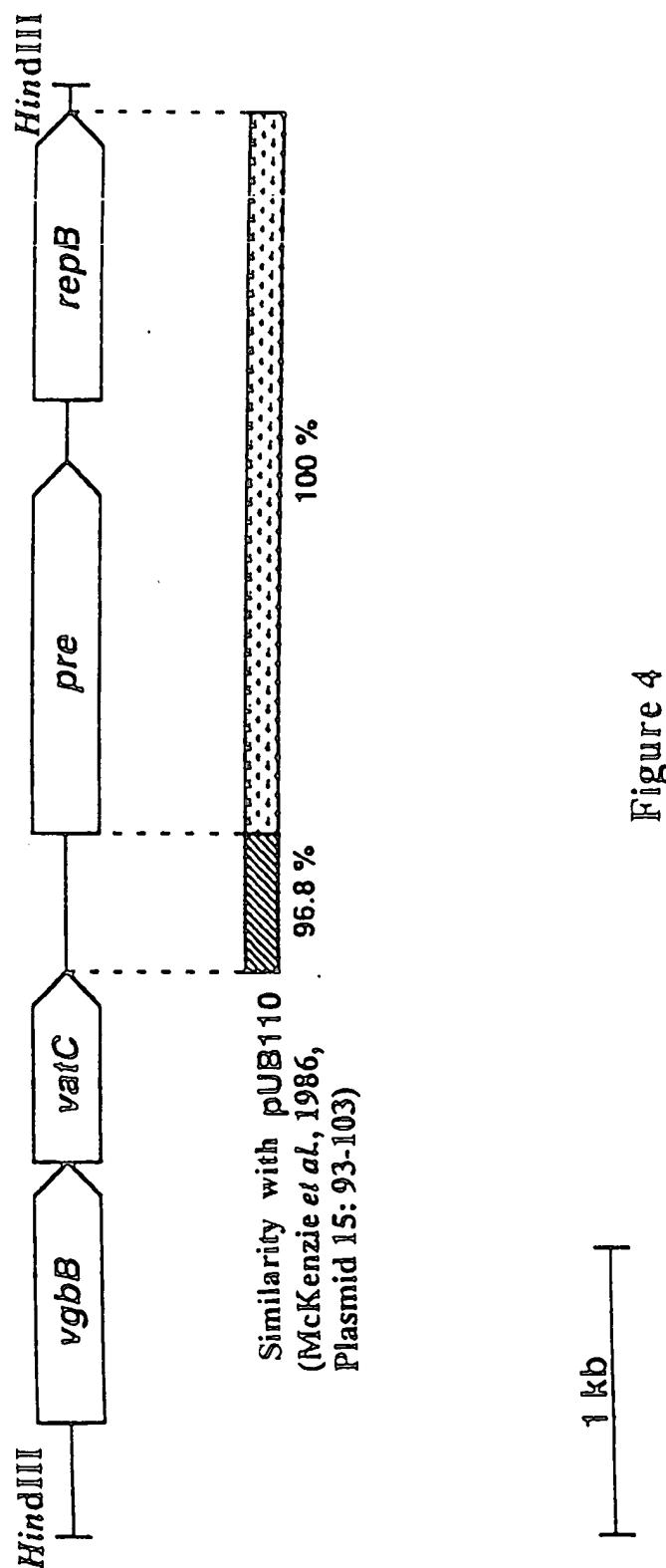


Figure 4

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PCR Primers

Figure 6A

Figure 6B

Figure 6C

5' -AAGTCGACTGACAATATGAGTGGTGG- 3'

Oligo II 5'-CTGCAGATGCCAACAGCATCGATATCC- 3'

vga B

Oligo VI 5'- catacgggalccacctttcc-3'

5' -AAGTCGACTGACAATATGAGTGGTGG- 3'

Oligo I

SUBSTITUTE SHEET (RULE 26)

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SEQ ID NO: 1

Seq vgaB

ATGCTTAAATCGACATGAAGAATGTAaaaaaATTATGCAGATAAATTAAATTAAATATAAA
 AAAGATTATAGTGGGGATAAAATAGGTATTGTAGGTAAGAATGGAGTTGGCAAAACACTTT
 TAATAAAAGGACTAATAGAGATTGACGAAGGAAATATAATTATAAGTGAaaaACACTATTAA
 TCTCAATTAGAAGAACCATAGTAAGATAATTGATGGAAATATGCTTCATAATTTCAGTTGA
 GTGGAATGACAATATGAGTGGTGGTAAAAAAACTAGATTAAACTAGCAGAGGATTGAGATC
 CTTTAATGCTCGTAGATGAACTACAGTAATTAGATATCGAAGGAATAGAGTTGATAACAAAT
 AAAGAGTACCGTGTATACTTTTGGTACTATCTCATGATAGAAATTTTTGTAGATCAGTTGTC
 TTTGAAATTGAAATGGATATATTAGAGAAATCATGGTAATTATAACAACTATATAAGCAAA
 TGCTTCTACGAAAGCAACAGAAGAATACGAAAAGTATAATTCTAAAGAAAGCAATTGGAGCA
 AAGCTAAAAGAGAATAAGGCGCAAGGAATGATTAAGCCCCCTCAAAACAAATGCGACATCTGA
 AATATGAAAATGCAACATGCTACTAAACAAAAAGATGCAAGAAATACGAAATCCTGGAAA
 TAGATAAAATTAAATCATGAGAAAAAATAAGAGCTTCTTCTATTAAATGGATTACCTAAT
 CAATTCTATGGTCGCAATGTAATTAGTTAAAACCTATCTATAAAATTAAATAATCATTCT
 AGATGCTCATTGTCATTAAAGGTGGAGAAAAAGTTGCTATAATTGTAACATGGTGTACGAA
 CATTGTTGAAGCTGATTCTAGAAAAGTAGAATCAGTAATAATATCACCATCAGTTAAAATTGGA
 AGTCAAAACCTAGATGTTCTACAATCTCATAAAATCTATCTTAGAAAATCTATGTCACCTCCAT
 TGAAACAAATAGCAAGAATTGTTCTAGCAAGATTACATTATCTGCAATGATGTTCTATAAGAAA
 TTTTGAGTGGTGGAGAACAAATAAGGTTGCTTTGCCAAGCTATTGTTAGCGATTGTAATACA
 CTTGATGAAACCACAAACTATTGATATCGATGCTGTTGAGGCATTAGAAGAATTGTTAATTAC
 AGGTGTTGTTATTGCTTCCATGATAAAAATTATAACAAAACCTAGCTGAACAAATTGTTAA
 AAAATAATRAAGTGAaaaaATTGAAAGGAACATATATAGAAATTAAATTAAAGATAAAACCA
 AATACAAATGAAAAAGAACTCAAAGAAAAAGATGATACTAGAAATGCAAAATTCTCATCATTATT
 AATCTCAATGAAAGAAAATGAAAGAAAAACAAAGAATTAGATGAAAAGTACAATTGAAATTAA
 TGAAAAGCCTAAATAAAAATT

Seq vgb8

SEQ ID NO: 3

ATGAATTCTATTAGAGGAGTTAACCTGCTATTCCGATTCAAGTCCATACGGTATAACTTC
 AGACGGAAAGGTATGGTCAACACAAACATAAGGAAACAAATCAGCAGTCTAGATCAGAGTGTAA
 AAGAATTGAAAGTCTCTACCCCTGATGCTAAAGTGTGTTAATTGATCTCTACTTGGAGAC
 TTACAGAGAATGGTGCATAAAATCGAAAGCTCTAAAAAGGGCTTACGAAATATCC
 ACAGCCGGATTCTGGTCTTACCGAATAACGGAAAGGTCTAAATGGCGATATATGGTTACCCAT
 GAGATGTTAGGAAAGTTGACAGCTGATGGGACTATTGAAATATGATTGCTTACGAAATAAGGGA
 CCTGCTTTATTACTTGTGGATAACGCACCTGGTCTACGGAGAACCAAAATAATTCTAT
 GATTACAATACAGGGAAATTAGAAGAAATCTCTACCAACAAATGCAGGGCTCCAGTGGTA
 GTGGTAACGATGGTCACTCTGGTTGCAAAATTGCGCAACAAAATAGGTGAAATCCTAC
 GAGATTAGCGAATATGATATTCCAACCTCAAACGACGGTCCACACGGCTATAACCGCGGGGAAAAA
 AATAATGTTTACTGAAATGGGGGCAATCAAATCGGCAAGAATTACAAACGACAAAACATTCAAG
 AACTCTAAACAGAAAATGCGGAACCTCATGGTATACCTTGGAAAAGATGGATCGTATGGTTT
 AAATGTAATTGGGAAGCTGAAATTGAAACGAA

Figure 7

10/12

SEQ ID NO: 2

Seq varC

ATGAAATGGCRAAATCAGCAAGGCCCCAATCCAGAAGAAATATAACCTATAGAAGTAATAAAC
AATTTATTAACCATCTATAACAAAGCCAATATTTACTTGGGAATATTCTATTACGATAG
TGGTGAATCTTTGAAAGCCAAGTTCTTATCACTATGAATTGATTGGGATAAAGTAATATTA
TTTGTCTATTGGACCGGAACGACATTATAATGAATGGGCTAATCATCGTATGGATGGT
TTCCATTCAATCTTTCGGAAATGGTGGGAGAAGCATACCCCTACATTGGAAGACCTTCCTTA
TAACACGGAAATTGGGACCGATGTTGGATTGGACGAGATGTGACAATTATGCCCGGTAA
AACGGGGCTATTATTGAGCAGAAAAATCGGTTGTGACAAGAACGTTGATCCTTATTCA
ATCCTTCACGATTAAAGATAAGGTTTCAAGGAAAAAATCGCAGCATTACTAAAGTAAG
GGACCTAGAGATAGAGACGATAAAATATTGATTGCATCCTGAATGGTGTATAAAAAAG
AGAAGT

Figure 7 (cont.)

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SEQ ID NO: 4

VgaB

MLKIDMIVQVKKYYADKLILNIKELKIYSGDKIGIGVGRNGVGKTTLLKIK
 GLIEFIDEQNTIISSEVTTIKEYISQLEEPHSKIIIDOKYASIFQVENKWNDNM
 SGGEKTRFKLAEGPQDQCSMLVDEPTSNLIDIEGIELITNTFKEYRDTFL
 VVSHDRIPLDQVCTKIFEIENGYIREPIGNYTNYIEQKEMLLRKQQEEYE
 KYNSKRKQLEQAIKLIENKAQGMIKPPSKTMGTSESRIWIKMQHATKQIKM
 HRNTKSLETRIDKLNHVEKIKEPSIOMDLPNREQFHGRNVISSLQNLSIK
 FNNQFLWRDASFVIKGGEKVAIIGNNGVGKTTLLKILEKVESVIISP
 KIGYVSONLQSHKSILENMSTSIQDETIARIIVLHFYRNDVHCE
 INVLSGGEQIKVAPAKLPVSDCNTLILDPTNYLDIDAVEALELLITYE
 GVULFASHDCKVPIQNLAPQLLIENNKVQKFGETYIEYLKIKDKPKLNNTN
 EICELKEQOMILEMQISSLLSKISMEEENKELDSKYKLKLKELKSLNK
 NI

SEQ ID NO: 6

VgbB

MNFYLEEFNLSIPDSGPYGITSSEDGIVWFTQHICANKISSLDQSGRIKEF
 EVPTPDAKVMCLIVSSLGDIWFTENGANKIGKLSKICGGPT2YPLPQPDSC
 PGITEGLNGDIWFTQLNGDRIGKLTADGTIYEYDLPNKGSYPAFITLGSD
 NALWFTENQNNISIGRITNTGKLEYPLPTNAAFVGITSGNDGALWFVEI
 MGNKIGRITITGEISBYDIPTPNARPHAITAGKNSEIWFTEWGANQIGRI
 TNDKTIQEYQLQTNAAEPHGITFGKDGSVWPALKCKIGKLNLN

SEQ ID NO: 5

V28C

MKWQNQQGPNPSEEIYPPIECNKHVQPIKPSITKPNILVGHYSYYDSKDGES
 FESQVLYHYELIGDKLILGK7CSIQPGTTFIMNGANHMDGTFPFNLFG
 NGWEIHTPTLEDLPYKGNTRIGNDVWIGRDTIMPVGKLGNGAIIAKSV
 VTKNVDPY5VVGGNPSRLIKIRPSKEKIAALLKVRNWDLKISTINENIDC
 ILNGDIKKVRS

Figure 7 (cont.)

12/12

SEQ ID NO: 7 K S I L E N V **VgaB**
1795 5' – aaa tct atc tta gaa aat gtt – 3' 1815
SEQ ID NO: 11 g agc t **vgaB**

SEQ ID NO: 9 I M N G A N H R M
 S. ID NO: 13 1187 'a c a a 1213
 5' - ata atg aat ggg gct aat cat cgt atg -3' vatC
 t t a c g vatC

SEQ ID NO: 10 G N D V W I G **VatC**
SEQ ID NO: 14 1310 a t g t 1330
 5' - ggg aac gat gtt tgg att gga - 3' **vatC**
 a t a a t

Figure 7 (cont.)